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Developmental validation of a fully integrated sample-to-profile rapid human identification system for processing single-source reference buccal samples

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ABSTRACT

Short tandem repeat (STR) DNA typing is a global standard for human identification. Current practice involves highly trained forensic analysts, operating in a laboratory setting, using multiple instruments to process samples and analyze the data. Here, we report the developmental validation of a fully integrated and automated DNA profiling system, the RapidHIT® System, capable of producing up to five high quality STR profiles with full controls in approximately 90 min using PowerPlex® 16 HS RapidHIT chemistry. The system integrates all sample handling steps: starting from lysis of cells on buccal swabs or other buccal sample types through DNA extraction, normalization, amplification,capillary array electrophoresis, detection, and integrated software analysis.

The results describe the developmental validation of the RapidHIT™ System for buccal samples processed with the DNA IQ™ extraction chemistry using a guandinium chaotropic agent and paramagnetic beads followed by amplification using a modified version of PowerPlex 16 HS chemistry (PowerPlex 16 HS RapidHIT chemistry), and capillary electrophoresis with manual review of genotyping data following interpretation guidelines. All processing from the buccal swab to generation and processing of the profile occurs on the RapidHIT platform.

Result are concordant with traditional methods, with 88% first pass success rates for both the CODIS and PowerPlex 16 loci. Average peak height ratios were 0.89 for buccal swabs. The system produces full profiles from swabs with at least 176 ng of saliva DNA. Rapid DNA identification systems will significantly enhance capabilities for forensic labs, intelligence, defense, law enforcement, refugee and immigration applications, and kinship analysis.

1. Introduction

Analysis of short tandem repeats (STR) is the reference method for human identification using DNA typing. STRs are repetitive regions of chromosomal DNA that are comprised of core repeat units of 2–7 nucleotides in length [1,2]. Developed initially by the Forensic Science Service in the UK [3], STR analysis is now routinely used in both casework and reference sample analysis in human identification, forensics, paternity, and kinship testing.

Current STR typing involves highly trained forensic analysts who use multiple instruments to process and analyze samples in a laboratory environment. The basic workflow for reference and casework known samples is (i) DNA extraction; (ii) amplification of

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the STR loci by multiplexed polymerase chain reaction (PCR) with fluorescently-labeled primers; (iii) dilution of the amplification products with a fluorescently-labeled DNA size standard; (iv) size-based separation of the fragments by capillary array electrophoresis with laser-induced fluorescence detection; and (v) analysis of the resulting electropherograms using genotyping software by expert forensic analysts to determine the allelic genotype at each locus [4].

Advances in DNA technology in recent years and the demonstration of the value of DNA to crime and insurgence fighting have led to a growing demand for DNA analysis of both reference and casework samples, and to the development of more rapid and efficient processing techniques that enhance the capabilities of forensic laboratories. Multi-lane capillary array electrophoresis analysis significantly increased the throughput of forensic laboratories [5], and "islands" of robotic platforms have been implemented to handle the increasing workloads without impacting processing times and laboratory human resources [6]. Nonetheless, the modular approach to automation usually requires manual intervention by highly skilled forensic scientists at each step to process the samples through the different methodologies and instrumentation [7]. The actual throughput remains limited by the lack of integration of the complete process. An alternative approach has been to integrate the entire workflow. Microchip capillary electrophoresis (CE) technologies have been developed [8] and automated [9]. The integration of the PCR and electrophoresis steps has been demonstrated [10,11] and the analytical stages of DNA processing have been combined into compact and integrated systems [12,13].

Full integration of all steps in the workflow from sample to answer has the potential to dramatically reduce sample analysis times and minimize sources of human error. DNA identification using a fully integrated system to process samples from buccal swabs to DNA profiles has been termed Rapid DNA. Several groups have achieved full integration of sample-to-profile systems [14–19]. The first developmental validation of a rapid sample-to-profile system has been completed for GlobalFiler®, a 24-plex chemistry, on the RapidHIT System [20].

This study describes the developmental validation [21] of a fully integrated sample-to-profile system that processes up to five samples with three controls simultaneously for rapid human identification using PowerPlex 16 HS RapidHIT chemistry (Fig. 1). The system can be loaded with samples in under five minutes and executes the complete DNA analysis process from lysis of cells through purification and PCR amplification to the generation of a called DNA profile in approximately 90 min. Profiles from the system are displayed and reviewed by forensic scientists and output as CODIS- or NIST/ANSI-compatible files. A Rapid DNA System with manual review by forensic scientists has been termed a 'Modified Rapid DNA System' [22]. The system is designed to be compatible with databases such as the U.S. National DNA Index System (NDIS), State DNA Index Systems (SDIS), Local DNA Index Systems (LDIS), the UK National DNA Database (NDAD), watchlists, and other databases.

Fig. 1. RapidHIT System with disposable cartridges for samples, controls, separation polymer, and buffer. The system can process five samples with three controls in approximately 90 min. The system is approximately 28″ wide, 28″ deep, and 19″ high.

As the developmental validation of the RapidHIT System was the first for a sample-to-profile system, the developmental validation was adapted from the SWGDAM guidelines [21] to include studies of the complete process starting with sample extraction and normalization. The resulting developmental validation establishes the performance of the RapidHIT System for reference buccal samples or casework knowns using a modified PowerPlex 16HS chemistry, denoted 'PowerPlex 16 HS RapidHIT', with automated profile generation and manual review of all profiles.

2. Materials and methods

2.1. DNA sample preparation.

Buccal swab samples were collected from volunteer donors using 3" cotton-tipped swabs from Puritan Medical Products Company (Guilford, ME). Each donor was instructed to swipe the inside of the cheek ten times and contribute swabs daily to generate aged swabs for stability studies. After buccal collection, swabs were returned to the same paper package labeled with the date and an anonymized identification number, then stored at room temperature in a file cabinet. Buccal swabs from all donors were used to generate a reference database.

DNA extraction of buccal swabs was performed by incubating the swab with 400 μL of lysis buffer (DNA IQ System, Promega, #DC6700) for 30 min at 70 °C. The swab was placed in a spin basket and a further 100 μL of lysis buffer added, followed by ambient centrifugation (Eppendorf, 5417 R) for 3 min at 25,000 rpm. Ethanol was added to a final concentration of 80%, and the sample centrifuged at +4 °C for 1 h at 25,000 rpm. The supernatant was aspirated, and 1.5 mL of 80% ethanol was added followed by +4 °C centrifugation for 30 min at 25,000 rpm. The supernatant was aspirated and the DNA pellet resuspended in 50 μL of TE buffer. The DNA sample was diluted 1:100 in TE buffer prior to quantification on the Applied Biosystems 7500 Real-Time PCR System. The lysates were used to obtain an STR profile as described below.

Positive control swabs, which were designated 1000F, were prepared from the human embryonic palatal mesenchymal (HEPM) cell line CRL-1486™ (ATCC, Manassas, VA). Cell culture optimization and scale up was performed under contract by Aragen Bioscience (Morgan Hill, CA) and cells were stored in 90% FBS, 10% DMSO at −80 °C. Cells were washed and resuspended twice in PBS buffer, quantitated using a Scepter Handheld Automated Cell Counter (Millipore, Billerica, MA), and brought up to a working concentration between 200,000 and 10,000,000 cells/mL. 50 μL of the appropriate cell dilution was added to swabs and air dried at room temperature overnight.

The DNA Profiling Standard SRM 2391c, produced by NIST (Gaithersburg, MD), was used to test the accuracy of allele calls against NIST certified genotypes. For testing on the RapidHIT System, DNA from components A, B, C, and D was added to the PowerPlex 16 HS RapidHIT pre-mix reagents at 1–2 ng/20 μL.

A set of sensitivity buccal swabs was prepared by collecting approximately 50 swabs with additional spittle from a single donor over a period of a week, and pooling them in TE buffer. The cells were spun down at 1000 rpm, +4 °C, for 10 min in a Sorvall RC-5B Refrigerated Superspeed centrifuge. The pelleted cells were rinsed in 25 mL TE buffer, re-centrifuged, and the cells resuspended in 20 mL TE buffer. A titration of the cell solution was made in TE buffer and 100 μL of each cell dilution was deposited on fresh

cotton swabs. Total DNA load was determined for each saliva dilution by performing DNA extraction and quantification.

DNA quantification was carried out using the Quantifiler® Human DNA Quantification Kit (Life Technologies, Carlsbad, CA, #4343895) on the Applied Biosystems 7500 Real-Time PCR System running Sequence Detection Software version 1.4 (Life Technologies) with 10 min of activation at 95 °C followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. DNA extracted from buccal swabs with the DNA IQ system was quantified with the beads present in the real-time PCR to mimic the results in the RapidHIT.

A set of mixture buccal swabs was prepared with known ratios of quantified saliva from a male donor and a female donor. The total combined DNA concentration and cell volume from the two donors was held constant for each mixture ratio.

Buccal samples were also collected using a Bode Buccal Collector (Bode®, P01D28) with five strokes on the inside of the cheek. Samples were dried overnight at room temperature. 6 mm buccal punch samples were transferred into the RapidHIT sample and control cartridges using Accu-Chek SoftClix lancets (Roche Diagnostics GmbH, Cat. No. 971).

The robustness of the extraction method to remove PCR inhibitors was challenged using four models of PCR inhibition – coffee, tobacco, hematin, and EDTA. Dilutions of each inhibitor were added to 1000F control swabs. Three replicates for each cell load and inhibitor dilution were performed. The inhibitors were prepared as follows: (1) brewed black coffee was purchased from Starbucks® and 2, 10, 50, and 100 μL aliquots were pipetted directly onto 1000F swabs; (2) 2.5 grams of Grizzly Long Cut chewing tobacco (American Snuff Company) was mixed with 25 mL of water, ground in a pestle and mortar, and soaked for four hours. The tobacco slurry was stored overnight at room temperature and the next morning 2 μL, 10 μL, 50 μL, and 100 μL aliquots of the supernatant were pipetted onto 1000F control swabs; (3) a hematin stock solution of 2 mM was made by dissolving hematin (Sigma–Aldrich, St. Louis, MO) in 0.1 N NaOH and then diluted in sterile water to desired concentrations. 20 μL of each dilution (0.3 mM, 0.6 mM, 1.0 mM, and 2.0 mM) was pipetted onto 1000 F control swabs. For addition to swabs, 0.5 M EDTA (Ambion, AM9260G) was diluted in Ultrapure DNase/RNase-free distilled water (Life Technologies, #10977) to either 50 mM or 250 mM, and 20 μL was then pipetted directly onto a dry cotton swab containing 1000F cells immediately before use. These experiments were performed using three instruments.

For addition to the STR premix, 0.5 M EDTA was diluted in Ultrapure DNase/RNase-free distilled water and then pipetted directly into the STR pre-mix to final concentrations of 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM and 1.0 mM. 1000F control swabs with 25,000 or 100,000 cells were used to test effect of EDTA addition on generation of a DNA profile. Three replicates for each cell load and inhibitor concentration were performed.

2.2. PCR amplification, sample electrophoresis, and data analysis

Multiplexed PCR amplification reactions were prepared by combining 2 μL of PowerPlex 16 RapidHIT primer pair mix, 5 μL of master mix, and 13 μL of sterile water or sample to give a total reaction volume of 20 μL. For purified DNA or control DNA 2800M, 12 μL of sterile water, 2 μL of PowerPlex 16 primer mix, and 5 μL of master mix was combined to form the premix and then 1 μL of purified DNA (1 ng/μL) added.

Thermal cycling was performed on the GeneAmp® PCR System 9700 (Life Technologies) with a 96-well gold-plated silver block. Thermal cycling parameters used the 9600 emulation mode or max mode: enzyme activation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 s and annealing/extension at 58 °C for 20 s. A final extension step was performed at 60 °C for 1 min, followed by a final hold at 4 °C.

Following amplification, samples were prepared for capillary electrophoresis by combining 1 μL of either PCR product or allelic ladder with 10 μL of a formamide/ILS cocktail (10 μL of Hi-Di™ Formamide, and 1 μL of ILS 600, Promega, #DG1071). Samples without added formamide were denaturated for three min in a 9700 thermal cycler set to 95 °C, then immediately snap-chilled in a cooling block stored at −20 °C.

Separations were performed on a 16-capillary 3130xL Genetic Analyzer (Life Technologies) using the Dye Set F module and a 36 cm capillary array. Standard run conditions on the 3130xL Genetic Analyzer used the following parameters: sample injection for 5 s at 3 kV and electrophoresis at 15 kV for 1800 s in POP-4® Polymer (Life Technologies), with a run temperature of 60 °C.

The electrophoresis results were analyzed using GeneMarker HID v2.4.0 genotyping software (SoftGenetics) using the default analysis settings for PowerPlex 16 chemistry. If a sample yielded off-scale peaks it was rerun after decreasing injection parameters to 2 kV for 5 s. A peak amplitude of 50 RFU (relative fluorescence units) was used as the peak detection threshold when analyzing data from all capillary electrophoresis experiments.

2.3. RapidHIT human DNA identification system

The RapidHIT System (IntegenX, Pleasanton, CA, P/N 10,005) is a fully integrated sample-to-profile DNA identification system (described in Supplemental materials). Operators interact with the RapidHIT System using a 10-in., 1024 × 768 pixel touchscreen. Detailed operation and additional system information is available in the RapidHIT user manual [23]. In brief, to operate the system, the operator selects the protocol to be run and is prompted to load the cartridges and up to five buccal swabs. The operator presses the run button and the instrument then prepares and performs STR analysis on the buccal swabs.

The RapidHIT System uses a single-use kit (IntegenX, 400,049) containing a sample cartridge and a control cartridge for sample preparation, an anode cartridge containing a linear polyacrylamide separation gel with a dynamic wall coating agent, and a buffer cartridge for CE separation. The sample and control cartridges (Fig. 2) are injection molded with an integrated fluidic device that uses externally actuated, pneumatically-driven valves and pumps [24] to transport samples and mix fluidic streams [25]. The sample cartridge processes up to four swabs, and the control cartridge processes one swab along with three controls: positive amplification control, negative amplification control, and an allelic ladder.

The RapidHIT instrument (Supplemental Fig. S1) is comprised of four main subsystems (described in more detail in the Supplemental material) which process the samples using the parameters shown in Table 1. In brief:

a The sample preparation subsystem operates the sample and control cartridges, employing pneumatics to provide motive force to the liquid in the cartridges, to process samples through lysis, extraction, purification using the DNA IQ System (Promega, #A8251); PCR amplification using PowerPlex 16 HS RapidHIT chemistry, which has the same hot start Taq enzyme and identical primer sequences to the NDIS approved PowerPlex® 16 HS (personal communication, Douglas Storts, Head of Research, Promega Corporation); and addition of internal size standard (ILS 600, Promega, DG1071).

b The separation subsystem receives the STR-amplified samples or allelic ladder mixed with the size standard from the sample and control cartridges, prepares the eight separation capillaries for use, injects the separation polymer from a disposable anode cartridge into the capillaries, transfers and injects the STR

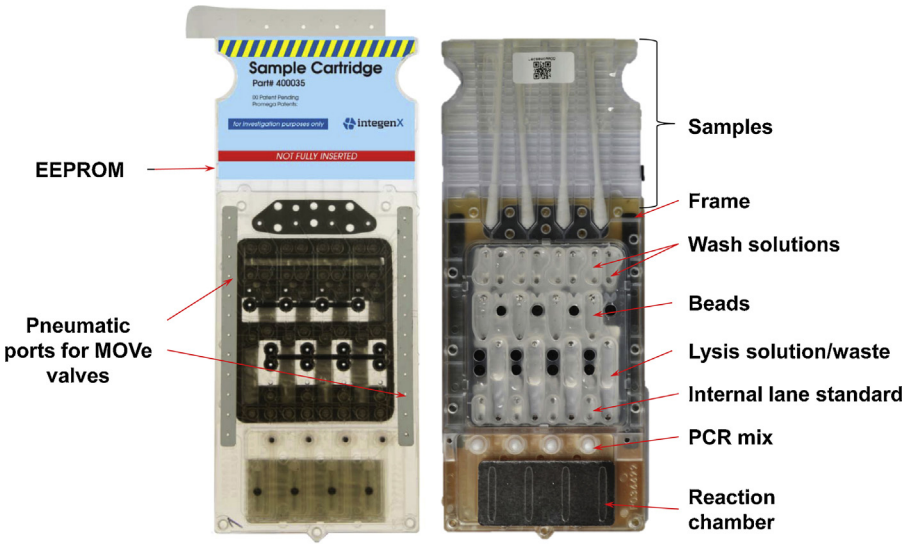

Fig. 2. Sample cartridge with major parts labeled. The wash solutions, beads, lysis solution/waste and the internal lane size standard are held in a reagent pack.

amplified samples into the capillaries using buffer supplied from the disposable buffer cartridge, performs a capillary array gel electrophoresis size separation of the labeled DNA, and finally cleans and processes the capillaries to ready them for the next sample run.

c The detection subsystem interrogates the fluorescently-labeled STR products as they traverse the detection window in the capillaries. The detection subsystem uses a 20 mW, 488 nm solid state laser (OBIS 488–20 LS, Coherent, Santa Clara, CA) and a cam-driven lens that scans over the eight capillaries at a rate of 5 Hz. Detection is with a CCD detector (Critical Link, Syracuse, NY, H7031–0906).

d The control and analysis subsystem runs an embedded computer equipped with 4 GB of RAM and a 120 GB solid state hard drive with an Intel® Core™ i5 processor running a secure version of Windows® 7 (Microsoft, Redmond, WA). DevLink™ software (Silicon Valley Scientific Inc., Pleasanton, CA) controls the hardware by executing script commands that operate the instrumentation and the MOVe™ valves on the cartridges. The software captures image data from the CCD and other readings from sensors, and processes and analyzes the data (described in detail in Supplemental materials, Supplemental Figs. S2 and S10, and Supplemental Table S1).

3. Results and discussion

A fully integrated sample-to-profile DNA identification system has been developed and developmentally validated for the analysis of buccal samples. The system is easy to use: the operator inserts single-use cartridges, up to five buccal swabs, inputs sample identification information, and presses run. The sample preparation, analysis, and data processing are automatically performed. The electropherograms and genotyping calls are then reviewed manually.

In the following sections, the performance of the overall system is reported and the operating parameters in the standard reference protocol are validated for each process of the system: Table 1 shows the standard conditions. Population studies were not conducted since the PowerPlex 16 HS RapidHIT primer sequences are identical to PowerPlex 16 HS (Doug Storts, personal communication). Boundary conditions on either side of setpoints were tested to demonstrate sufficient tolerance to small variations in parameters such as concentration, temperature, volumes, etc., to avoid process failure. The system has highly reproducible performance: 16/16 possible loci were called with a 88% first pass success rate of all buccal samples processed and with 100% concordance for all full profiles. (Table 2).

Table 1
Standard RapidHIT process.

Sample cartridge	Lysis	Lysis volume	0.5 mL
		Lysis temperature	70 °C
	Bead purification	Bead amount	30 µg
		Bead incubation time	3 min
	PCR	PCR reaction volume	20 µL
		PCR cycles	29
		PCR conditions	6 s at 98 °C
			30 s at 59 °C
			10 s at 70 °C
			Final extension, 4 min at 61 °C
	ILS	ILS volume	100 µL
RapidHIT instrument	Separation and detection	CE injection	10 sec at 5000 V
		CE separation	24 min at 9100 V
	Analysis	Data analysis	TraceAnalyzer
		Genotyping	GeneMarker® HID v2.4.0
		Review	Manual

Table 2
Success rate of buccal samples with manual review.

Metric	Success rate[a]	Number of samples
Buccal samples with fully correct PowerPlex 16 profile on first pass	88%	219/250
Positive controls with fully correct profile	100%	37/37

[a] Success rate is the percentage of samples that had full profiles.

3.1. PCR-based studies

3.1.1. Cell lysis and DNA purification on the RapidHIT.

As the RapidHIT System is an integrated system, the validation results for the first steps in the process – cell lysis and DNA purification – are discussed. DNA-bead binding using DNA IQ reagents has been tuned to capture a small fraction (~0.5%, data not shown) of the released DNA; buccal swabs typically contain a total of 100–1,500 ng of genomic DNA [26], which yields 0.5 ng–7.5 ng of purified DNA. The STR chemistry on the RapidHIT System performs well over an input DNA range of 0.5–10 ng for the PCR amplification step (see below).

A series of experiments (Fig. 3) were conducted to validate the lysis, DNA extraction, and purification steps on a RapidHIT System. A range of conditions were tested for impact of lysis buffer volume, lysis temperature, bead concentration, and capture time. Additional variables, including timing of processes, volumes of reactions, reagent optimization, etc., had previously been optimized during the development process. These experiments used the control cell line 1000F added to the swabs at three different quantities of cells: 10,000, 50,000, and 500,000 cells, covering the range of cell load found on typical buccal swabs (data not shown).

The impact of lysis buffer volume on the analysis of samples on the RapidHIT System was tested (Fig. 3A) using cartridges with lysis buffer volumes of 300 μL, 500 μL, and 700 μL and with 1000F control swabs at 10,000 and 500,000 cells. All six conditions generated concordant full profiles. The average normalized peak heights of alleles (Fig. 3A) were similar, within experimental error, for all three lysis volumes. The standard 500 μL lysis buffer volume accomodates the amount of buffer a dry swab can absorb while fully immersing the swab head in liquid during the lysis process.

During lysis, the system heats the samples in the lysis buffer to produce a controlled average temperature of 70 °C. The effect of lysis temperature was tested at 65, 70, and 75 °C with 10,000 and 50,000 cells per swab (Fig. 3B). Concordant full profiles were obtained on all samples. In general, the lower lysis temperature of 65 °C resulted in lower peak heights, compared to the standard temperature of 70 °C, while the higher temperature of 75 °C showed mixed results, with higher peak heights for 50,000 cells but not for 10,000 cells. The STR profile balance was not affected at

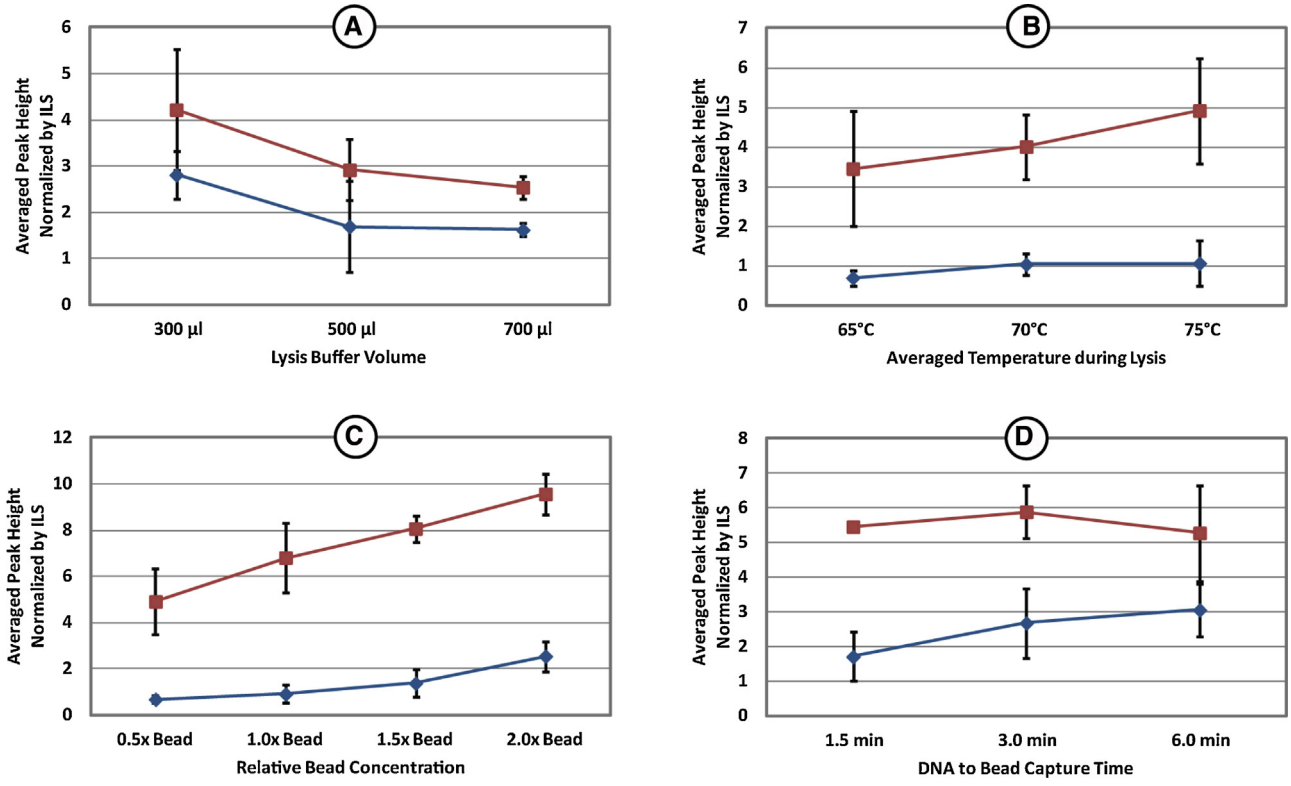

Fig. 3. Boundary testing of sensitivity of extraction to buffer volume (A), and lysis temperature (B) and of DNA purification to bead concentration (C) and capture time (D) were measured. Each data point was run in triplicate on a single instrument and is plotted as the mean ± standard deviation (S.D.) of the average STR peak height normalized by dividing the average peak height of the STR peaks by the average peak height of the ILS peaks from that sample (panels A, C, D: ◆10,000 1000F cells ■500,000 1000F cells; panel B: ◆10,000 1000F cells, ■50,000 1000F cells).

either low or high temperature. The standard lysis temperature of 70 °C was shown to be optimal and any deviations due to, for example, operating the system in different environmental conditions, should be well tolerated for reference samples.

The sensitivity of the DNA purification to the amount of paramagnetic beads was assessed (Fig. 3C). Cartridges were run with the 0.1, 0.2, 0.3, and 0.4 mg/mL bead concentrations (0.5x, 1.0x, 1.5x, 2.0x) using 10,000 and 500,000 1000F cells on swabs. 100% concordant full profiles were achieved in all samples with all four different bead concentrations. Reducing the bead concentration by half led to 25% lower overall signal intensity, while increasing the bead concentration increased the overall signal intensity by more than 50%. However, at both 1.5x and 2.0x bead concentrations, some – A peaks were observed for the 500,000 cell 1000F swabs. The 0.2 mg/mL bead concentration was shown to be optimized for reference swab samples without overloading the PCR reactions.

The sensitivity of the DNA purification to the bead incubation time was determined using 10,000 and 500,000 1000F cells on swabs (Fig. 3D) with capture times of 1.5, 3, and 6 min with intermittent air bubbling through the solution to promote mixing. When the capture time was reduced to 1.5 min., the overall signal intensity dropped slightly, presumably due to less DNA being captured onto the beads. A capture time of 6 min yielded slightly higher overall signal intensity for the low input swab of 10,000 cells, but a small drop in signal intensity for the high input swab of 500,000 cells, perhaps due to bead clumping or bead loss at high DNA inputs. Typical buccal swab samples have DNA quantities between the low and high 1000F swabs used here, therefore the 3 min capture time was shown to be optimal for reference samples.

3.1.2. Removal of inhibitors.

The ability of the system to effectively purify genomic DNA from inhibitors of STR amplification was measured. Four potential inhibitors, including those most likely present during buccal swabbing, were added directly to the 1000F positive control swabs prior to analysis in a RapidHIT System (Fig. 4).

Hematin is a common inhibitor of STR amplifications due to its presence in blood samples [27,28]. In benchtop tests, the PowerPlex 16 HS chemistry has been shown to tolerate up to 200 μM of hematin in the amplification reaction without any compromise in signal intensity or profile balance (data not shown). Four different concentrations of hematin solution were tested on the RapidHIT System: 300, 600, 1000, and 2000 μM, with 20 μL of each added to the 1000F control swabs containing either 10,000 or 50,000 cells (Fig. 4A). Concordant full profiles were obtained for all samples in all four runs. Overall signal intensity and STR profile balance were not affected in the range of hematin concentrations tested: this showed the system was effective in removing hematin prior to amplification. Hematin concentration in blood samples is estimated to be around 300 μM, thus the highest hematin concentration of 2000 μM tested here is well above the biologically relevant concentration.

The effect of adding brewed coffee (Fig. 4B) or an extract of chewing tobacco (Fig. 4C) to dried swabs containing 10,000 or 50,000 1000F cells was measured. No significant inhibitory effect was observed at any level of either inhibitor tested and full

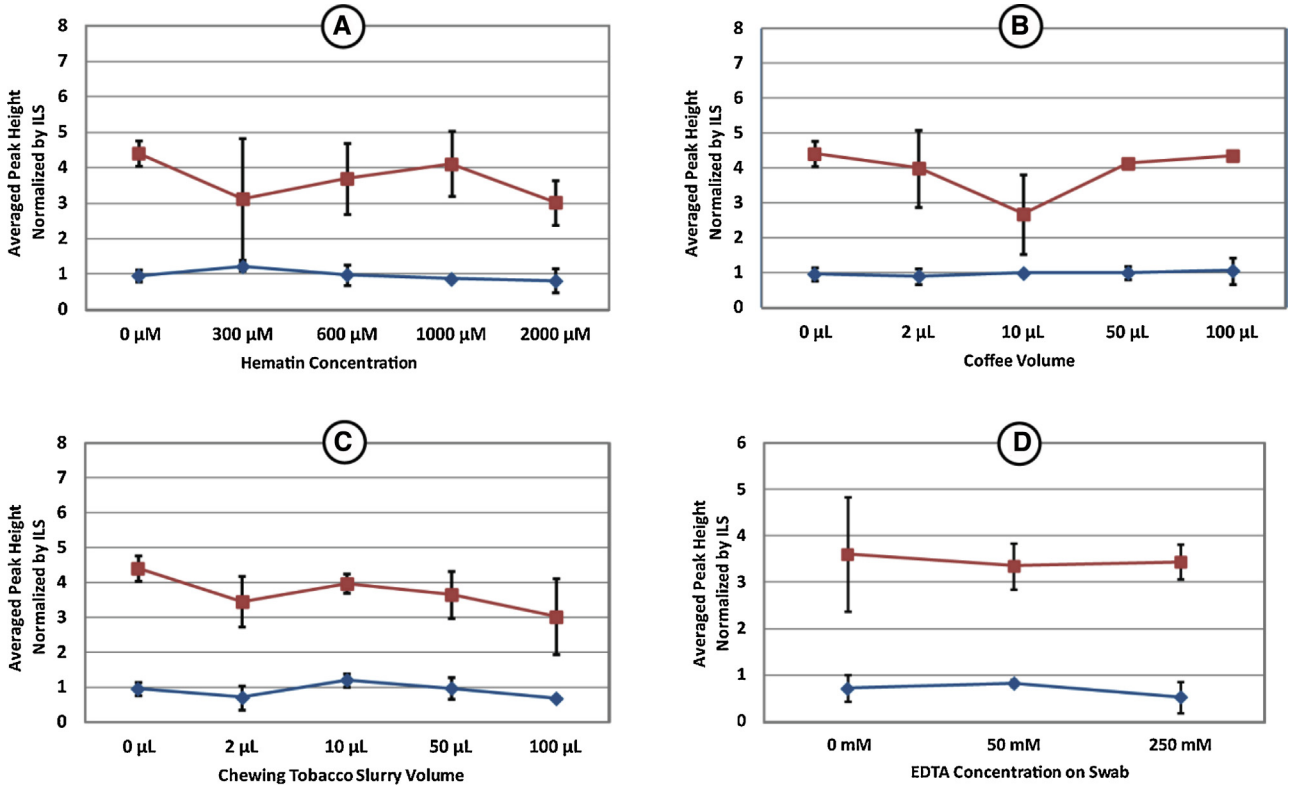

Fig. 4. Effect of inhibitors on peak heights. Hematin (A), coffee (B), mint tobacco slurry (C), and EDTA (D) were added to swabs and tested in the system. Each data point was run in triplicate on a single instrument and is plotted as the mean ± S.D. (◆10,000 1000F cells, ■50,000 1000F cells).

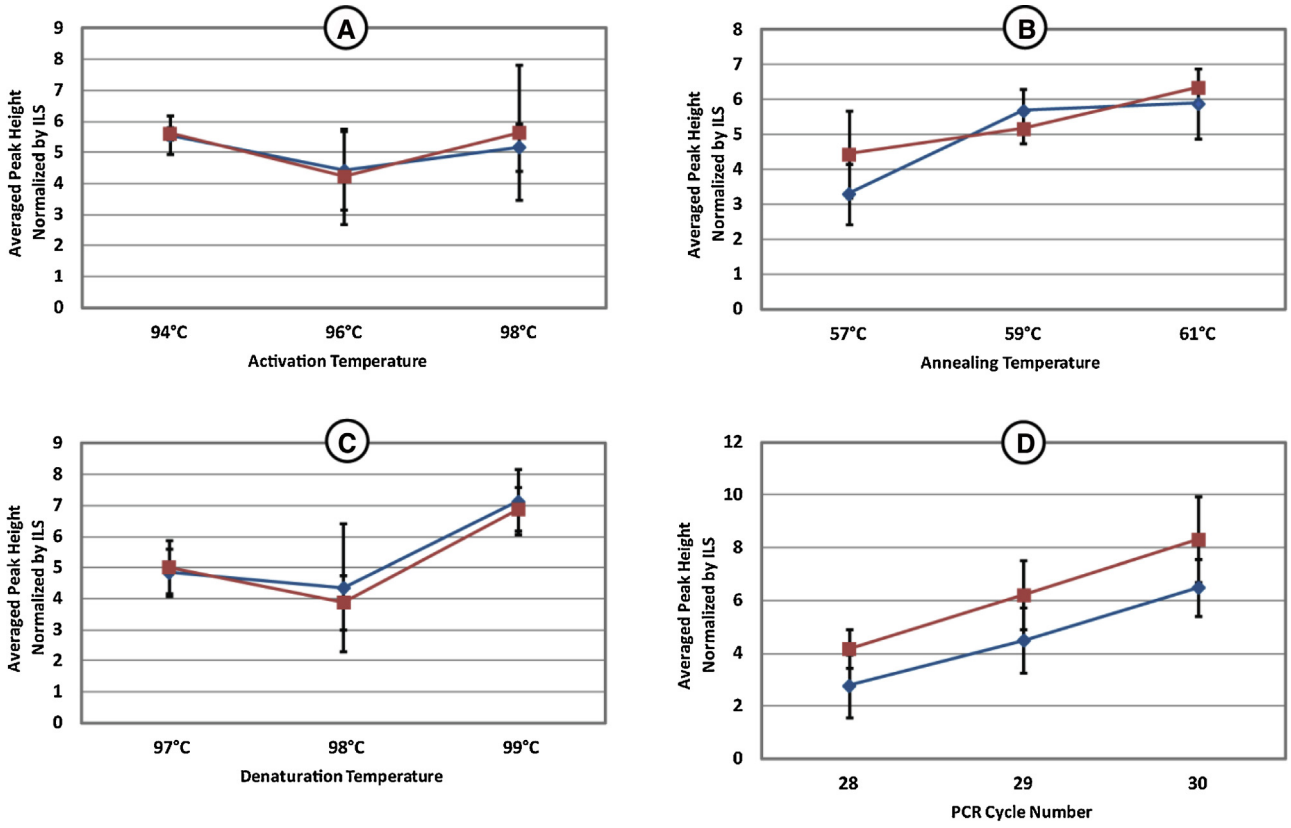

Fig. 5. Effects on PCR peak heights of activation temperature (A), annealing temperature (B), denature temperature (C), and cycle numbers (D). Samples were run in triplicate and are plotted as the mean ± S.D. ◆100,000 1000F cells, ■500,000 1000F cells).

concordance for all samples in all ten runs was obtained (five coffee, five tobacco). The STR profile balance and peak height show no correlation to the amount of coffee or tobacco on a swab.

The inhibitory effect of EDTA, a cation chelator, was also studied by adding 20 μL of a 50 mM or 250 mM EDTA solution to the 1000F swabs with 10,000 or 50,000 cells (Fig. 4D). The EDTA concentrations are orders of magnitude higher than the maximum EDTA concentration of ~2 mM in food approved by the FDA [29]. Full concordance of each triplicate sample was obtained. The impact of EDTA on peak height and profile balance was minimal, and demonstrates the system can effectively remove EDTA contamination.

3.1.3. STR amplification reaction conditions

The PCR reaction conditions were tested over a range of (i) enzyme activation temperatures, (ii) DNA denaturation temperatures, (iii) primer annealing temperatures, (iv) PCR cycle numbers (Fig. 5), and (v) with addition of EDTA (Supplemental Fig. S3) and (vi) final extension times (data not shown). In all studies, 1000F swabs were used with either 100,000 or 500,000 cells added. For reference, standard PowerPlex 16 HS conditions for the 9600 use a three step PCR (described in the Supplemental materials).

The PCR activation temperature is set to 96 °C. The temperature was varied by 2 °C above and below the 96 °C setpoint and its effect on 1000F samples was tested. 100% concordance was achieved at all three activation temperatures for all 18 samples, with no systemic effects on signal intensity or profile balance, (Fig. 5A) showing that small alterations in activation temperature did not negatively effect results.

The standard PCR annealing temperature is set at a 59 °C setpoint. Three setpoints were used, 57, 59, and 61 °C with triplicate samples of 100,000 and 500,000 1000F cells (Fig. 5B). Full concordance of 18 samples was obtained at all three setpoints.

The standard PCR denaturation temperature is set at 98 °C for 6 s. Varying the temperature up and down 1 °C from the set temperature had no effect on concordance, with all three temperatures generating 100% concordant full profiles. The 99 °C setpoint had higher average peak heights (Fig. 5C, bottom left) but slightly worse profile balance than the standard 98 °C setpoint. At the 97 °C setpoint, the TH01 locus, known for its secondary structure, exhibited lower peak heights than at 98 or 99 °C. This set of experiments confirmed the selection of the 98 °C setpoint for denaturation.

Final extension times of two, four, and six minutes were tested (data not shown). Full concordance was obtained in all 15 samples. Four min was chosen as the standard to reduce PCR cycling time while still generating high quality, full length amplification products.

To test PCR amplification cycle number, 100,000 and 500,000 cells of the 1000F control swabs were used in triplicate at 28, 29, and 30 cycles of PCR amplification (Fig. 5D). Full concordance was found in all conditions, except an FGA allele dropout was present in the on-cartridge, positive control sample amplified for 28 PCR cycles. Increasing the cycles to 30 led to a significant number of – A peaks in the product, while reducing to 28 cycles resulted in lower signal. The standard PCR cycle number was set to 29 cycles to achieve the optimum signal and profile balance for buccal swab samples.

The tolerance of the PCR amplification to EDTA was studied by spiking EDTA into the STR premix at a range of final concentrations from 0.1 mM to 1.0 mM and measuring the concordance and peak heights of alleles (Supplemental Fig. S3). Full concordant profiles were obtained with up to 0.5 mM of EDTA in the STR premix. No inhibition was seen at 0.1 mM and 0.25 mM EDTA, while at 0.5 mM EDTA peak height and profile balance started to deteriorate. Allele dropouts, specifically Penta E and FGA, were observed with 0.75 mM EDTA. At 1.0 mM EDTA, STR amplification was completely inhibited and no amplification product was obtained in any of the samples.

3.1.4. Stutter

Stutter was calculated from 150 samples. Stutter peaks were calculated at one repeat unit shorter than the true allele by dividing the peak height of the stutter peak by the true allele peak height. Fig. 6 shows the average percent stutter for each locus in PowerPlex 16 HS RapidHIT. The stutter range across all loci measured on the RapidHIT System (1.5%–7.5%) is very similar to that reported for PowerPlex 16 HS validation (3%–10%) [30], with TH01, Penta E, Penta D, and TPOX showing the lowest average stutter in each system.

3.2. Precision and accuracy

3.2.1. Precision

Sizing precision was measured by injecting allelic ladder into capillary lanes 1–5, 7 and 8 for a total of 42 individual injections. The allelic ladders were sized within the GeneMarker software using the ILS 600 size standard and the Local Southern method; the standard deviation of fragment size from all runs was calculated for alleles in the ladder.

Fig. 7 shows the sizing precision for each locus. The median range of standard deviation across all loci is in the range 0.07–0.11 bp. Loci with longer fragments tend to have higher standard deviation values. 99% of all fragments have a sizing precison less than 0.15 bp.The exceptions are two outlier points at the D18 locus. All data points had a sizing precision less than 0.17 bp which ensures alleles very rarely size outside of ±0.5 bp, which is set as the bin size.

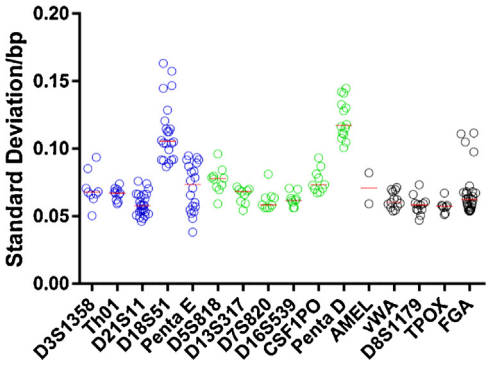

Fig. 7. Standard deviation of sizing accuracy for each locus, measured on three different instruments, performing two runs on each systemwith median values highlighted by the red line.

The resolution on the RapidHIT System was measured using the cross-over plot methodology [31]. Up to the cross-over point, measured in bp, visual inspection of the electropherogram allows the manual reviewer to clearly see the presence of two distinguishable peaks which differ by a single bp. The resolution at the cross-over point is 0.59, calculated from the formula $R = \Delta x/(2\sigma_1 + 2\sigma_2)$, where Δx is the migration time difference between peaks and σ is the standard deviation of the peak width. For the PowerPlex 16 HS RapidHIT multiplex chemistry single base resolution is required up to 474 bp. Fig. 8 shows an example of a sample with two Penta E alleles that differ in size by a single base (432 bp and 433 bp). The cross-over point for this electropherogram was measured at 475 bp.

3.2.2. Accuracy of standard samples

To assess accuracy and concordance of allele designation, the NIST Standard Reference Material (SRM) 2391c components A, B, and C were run in triplicate on a single RapidHIT System and component D was run a single time (Supplemental Table S2). The components were

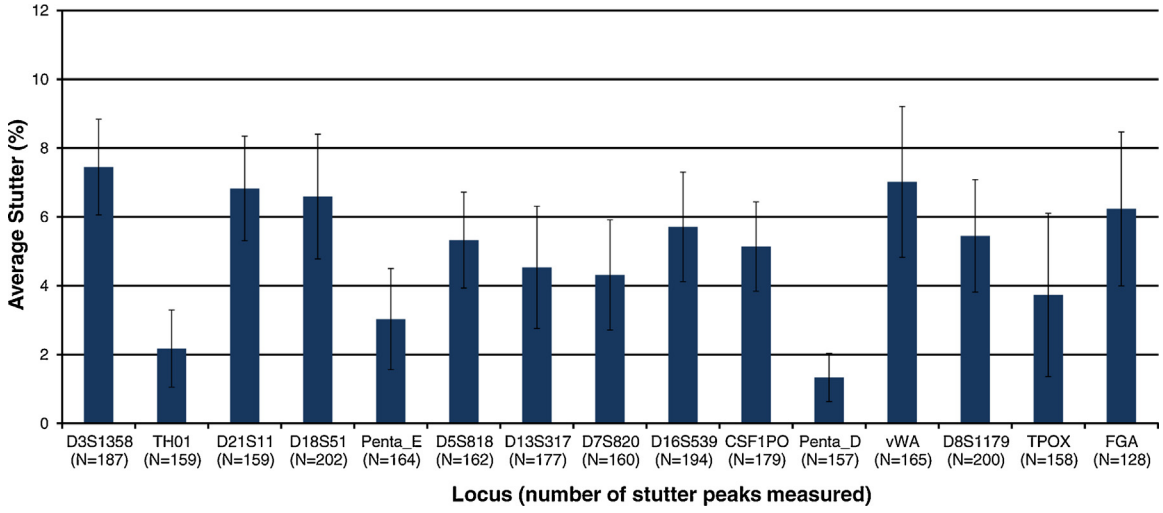

Fig. 6. Average stutter for each STR locus. The error bars represent the standard deviation from 150 samples.

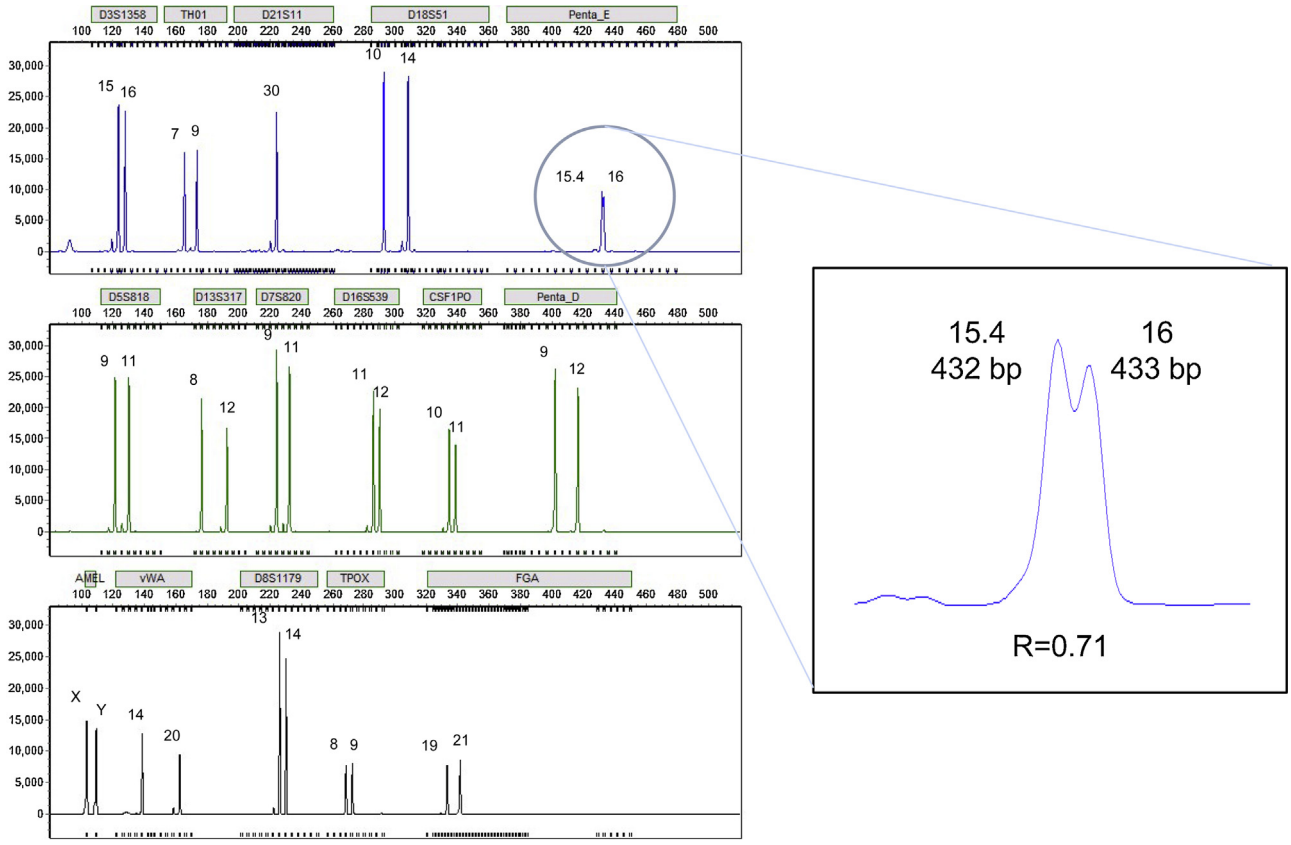

Fig. 8. Separation of 15.4 and 16 Penta E alleles that differ in size by a single base pair.

added to the STR pre-mix vial of the sample cartridges. All RapidHIT profiles generated were fully concordant with the certified reference profile.

3.2.3. Concordance

Overall system performance was measured using swabs from 150 individual donors run in singlet using five RapidHIT Systems and an additional 100 samples (ten samples of different ages from ten individuals) were analyzed on a single RapidHIT; the samples included individuals with microvariants and trialleles. Profiles from all samples were compared for concordance with reference profiles generated using standard bench top genotyping with PowerPlex 16 HS RapidHIT chemistry or for the ten replicates of ten samples using PowerPlex 16 HS analyzed on a 3130xL Genetic Analyzer. The swab age ranged from 0 days (fresh) to 569 days old. After manual review and interpretation, all alleles called were concordant.

In addition, to confirm the PowerPlex 16 HS RapidHIT chemistry produced the same genotypes as the NDIS-approved PowerPlex 16 HS, 84 of the 150 samples were also genotyped on the bench using the NDIS-approved PowerPlex 16 HS chemistry: identical genotypes were obtained. This small study established the concordance between PowerPlex 16 HS and PowerPlex 16 HS RapidHIT which have identical primers and polymerase.

3.2.4. Success rate

The system had an 88% (219/250) first pass, full profile success rate for the 13 CODIS loci as well as for the 16 PowerPlex loci. All positive control samples generated accurate and complete profiles.

3.2.5. Peak height ratios

Fig. 9 shows the peak height ratios (PHR) for all heterozygous loci in the 150 singlet samples. The median PHR across all 16 loci for 1,722 samples was 0.89 which compares favorably with values reported for reference samples analyzed as part of the PowerPlex 16 HS developmental validation [30]. No relationship of PHR with age of swab was observed for swabs up to 6 months old.

3.3. Analysis of buccal swabs with Bode collectors

To assess the performance of the RapidHIT System with a range of sample substrate inputs, a protocol for buccal samples for Bode

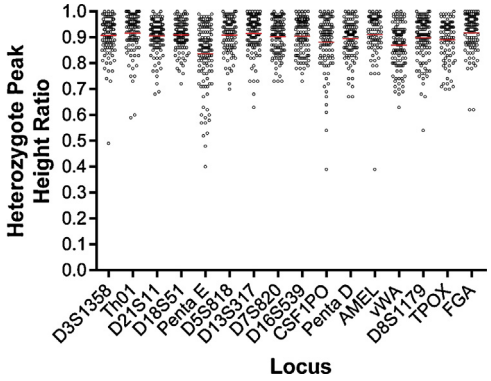

Fig. 9. Heterozygous peak height ratios from 150 samples processed on the system by loci with median values highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

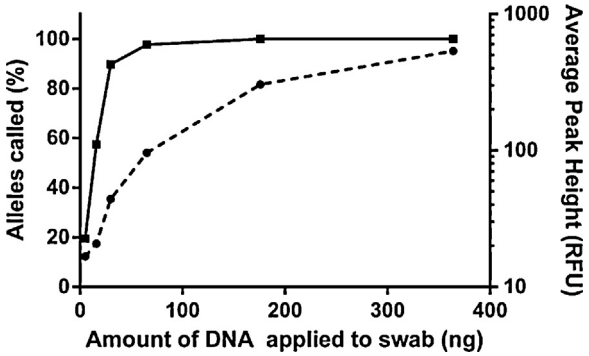

Fig. 10. Sensitivity of system detection for known DNA loads in saliva showing percentage of alleles called (■) and average peak heights (●). Average peak heights are scaled to reflect a maximum signal height of 29,000 RFU in the GeneMarker software. The average peak height for a profile is calculated from all detected alleles (average signal is used at heterozygous loci and signals are halved for homozygous loci).

(filter paper) collectors was developed and optimized with cycle numbers of 30, 31, and 32. Full concordance was found in all conditions for all samples (15 samples on one instrument, data not shown). No significant difference in peak height ratios was detected for any of the PCR cycle number conditions investigated.

3.3.1. Contamination

The system was tested for channel-to-channel contamination or crosstalk as well as run-to-run contamination over ten runs spread across three instruments (three consecutive runs on instruments one and two, and four consecutive runs on instrument three) by running buccal swabs and blanks in a sample/blank/sample 'checkerboard' pattern for a run and then for the next run running buccal swabs and blanks in a blank/sample/blank checkerboard pattern. Despite high signal from samples in channels 1, 3 and 5, no crosstalk was seen in the blank channels (Supplemental Fig. S4). Across all checkerboard runs, no evidence of sample-to-sample, or run-to-run carryover was found in any run except one called allele peak was found in one blank sample. In addition, all 37 concordance runs showed no alleles in the negative controls.

3.3.2. Sensitivity of electrophoresis to sample denaturation

Temperature is used to heat denature DNA samples on the RapidHIT System prior to electrophoresis injection and separation; no formamide is present in the sample. Denaturation of the sample was studied at 91 °C, 93 °C, and 95 °C. The sample spends approximately 20 s at elevated temperature as it passes through a heated denaturation region on the way to the cathode injection manifold. Inadequate sample denaturation can result in the presence of double-stranded DNA peaks in the electropherogram, which migrate faster than their single-stranded counterparts. Supplemental Fig. S5 shows a comparison of the ROX-labeled internal lane standard run at three different sample denaturation temperatures. The electropherograms show no evidence of double stranded DNA fragments across a broad range of fragment sizes including the longer fragments which are more likely to be impacted. The effect of pre-electrophoresis sample denaturation temperature was also examined in the context of control DNA samples amplified on the system. Two different levels (1 ng and 4 ng) of control DNA 2800M were amplified and subjected to the range of denaturation temperatures above. No systematic change in quality of the profile or the alleles called in the samples was observed as a function of the denaturation temperature (data not shown). The standard temperature for heat denaturation prior to electrophoresis was set to 95 °C which allowed the system to be operated without formamide.

3.4. Sensitivity studies

A RapidHIT System sensitivity study was performed to measure sample concordance and average peak heights for a set of control swabs made using a serial dilution of saliva. The total DNA load for

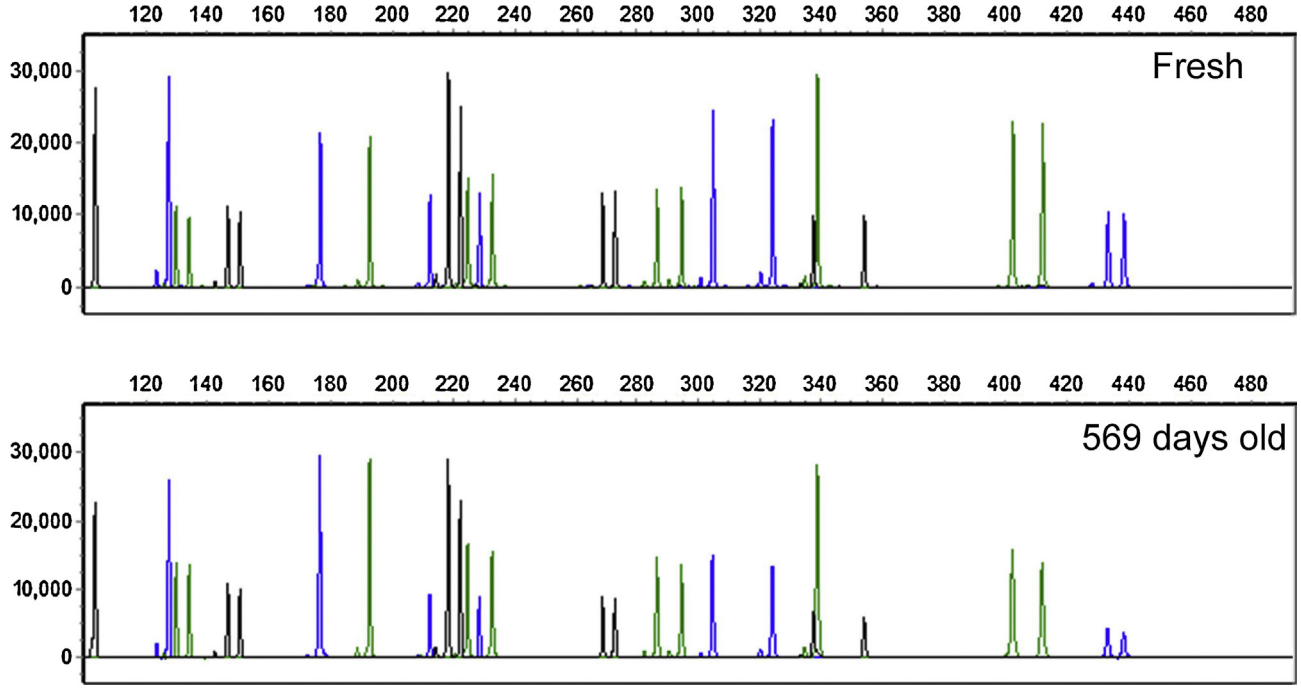

Fig. 11. Electropherograms of fresh and 569 day old swabs from the same donor yield the same profiles.

each saliva dilution on the swab (5, 16, 30, 65, 176, and 364 ng) was quantified using qPCR. Each dilution series was run on three different instruments. The average number of alleles called and peak heights were calculated for each swab dilution (Fig. 10). Swabs with at least 176 ng of saliva DNA gave concordant full profiles; 57% of alleles were detected with 16 ng of DNA on the swab. Alleles at the vWA locus, followed by FGA, are the first to drop out of the profile as the amount of saliva DNA is reduced and, in general, alleles in the TAMRA dye channel are more susceptible to drop out because this fluorescent dye has the weakest emission on the RapidHIT System.

To determine the sensitivity of the amplification and detection, control DNA was added directly into the PCR master mix and run in a single cartridge set. Input DNA in the range 0.5–20 ng gave concordant full profiles; 56% of alleles was detected at 50 pg of total DNA (Supplemental Fig. S6).

Adding the DNA directly to the STR reactions gave more sensitive detection than the saliva studies, primarily due to the estimated DNA extraction and normalization efficiencies of 0.5% for saliva and other samples in this protocol. Adjusting for differences in PCR cycle number, sensitivity of amplification and detection on the RapidHIT compares favorably with data reported for PowerPlex 16 HS validation [28].

3.5. Stability studies

3.5.1. Effect of age of swab

The performance of the system was tested with swabs aged up to 569 days. Using the standard protocol, both fresh buccal swabs and swabs aged for 14 days, 1 month, 3 months, and 6 months produced very similar results (data not shown). Neither the peak heights nor the first pass success rate were significantly impacted by the age of the swab. All swabs generated fully concordant data (not shown) and comparable profile balance to fresh swabs.

Comparing electropherograms from fresh and 569 day old swabs, the 569 day old swab shows decreased peak heights in some of the longer alleles but was fully concordant (Fig. 11). These data demonstrate the RapidHIT has the ability to process either fresh or aged swabs.

3.5.2. Swabs can be re-processed

A study was conducted to test the capability to perform repeat runs of the same sample swab after processing on the RapidHIT System. At the end of each run, the five donor swabs were retrieved from the sample lysis chambers on the sample and control cartridges and stored in a sterile, 15 mL Falcon tube at room temperature until reanalysis, typically the next day.

Supplemental Fig. S7 shows that the average peak height per sample, corrected for zygosity, tracks with number of times the swab is re-run. As expected, the peak height decreases as the number of re-runs increases. However, since the typical DNA load on buccal swabs greatly exceeds the amount of DNA required for successful STR amplification, retention of even a small amount of DNA on the swab is sufficient to allow multiple re-runs. Although the average peak heights decrease with each run, the sample swabs still yielded 100% concordant data for each of the re-runs, except the 151M sample which gave a partial profile at the FGA locus the fourth time the swab was run. The study demonstrates the capability to generate full profiles from swabs that are re-run on the RapidHIT System.

3.5.3. Reproducibility across reagent lots

The reproducibility of the system was tested using different lots of RapidHIT reagent consumables. For the purpose of this study a single reagent lot comprised a sample cartridge, control cartridge, buffer cartridge, and anode cartridge, each with a specific date of manufacture. Three different reagent lots were assembled. The range of manufacturing dates spanned a period within the shelf life of each reagent. The results of the study (Supplemental Fig. S8) show the average peak height of all alleles in a run, corrected for zygosity, as a measure of the reproducibility had a coefficient of variation (CV) was less than 16% in all cases. Error bars on the graph

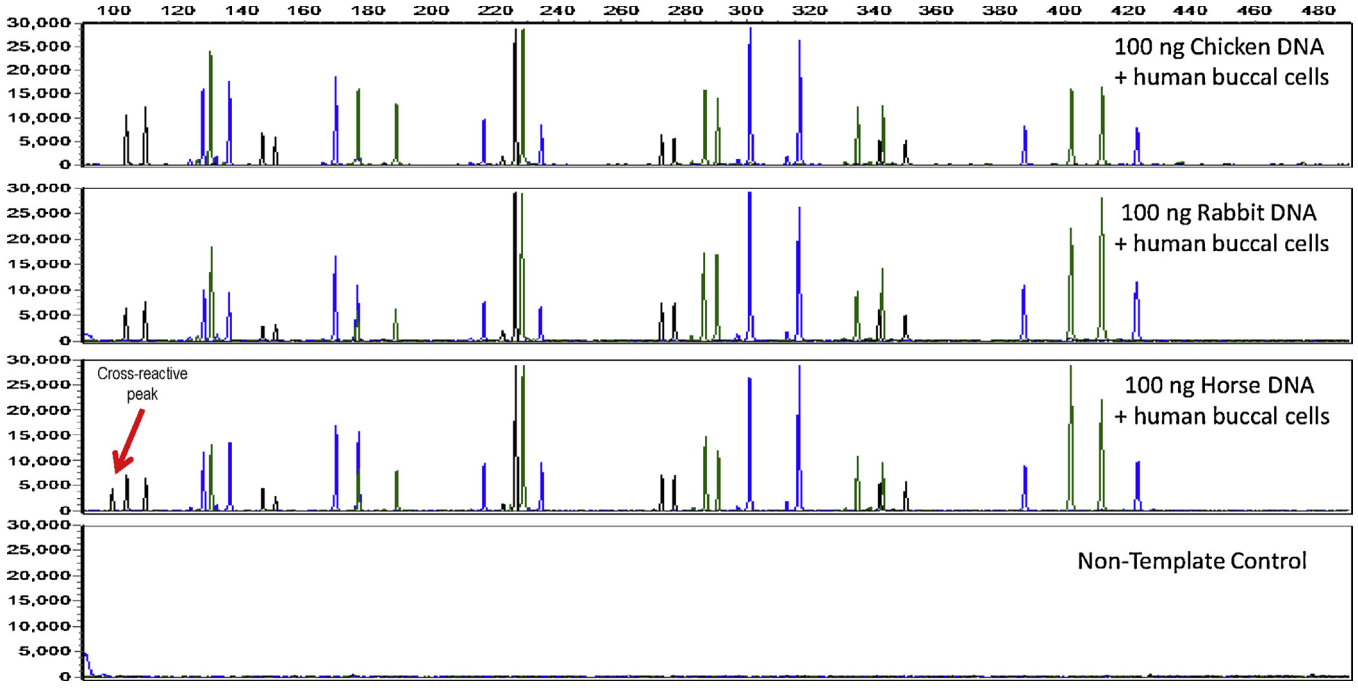

Fig. 12. Addition of DNA from chicken, rabbit or horse did not interfere with generation of a concordant genotype from human buccal cells. A cross-reactive peak from horse (red arrow) migrates below the amelogenin marker range and does not interfere with result. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

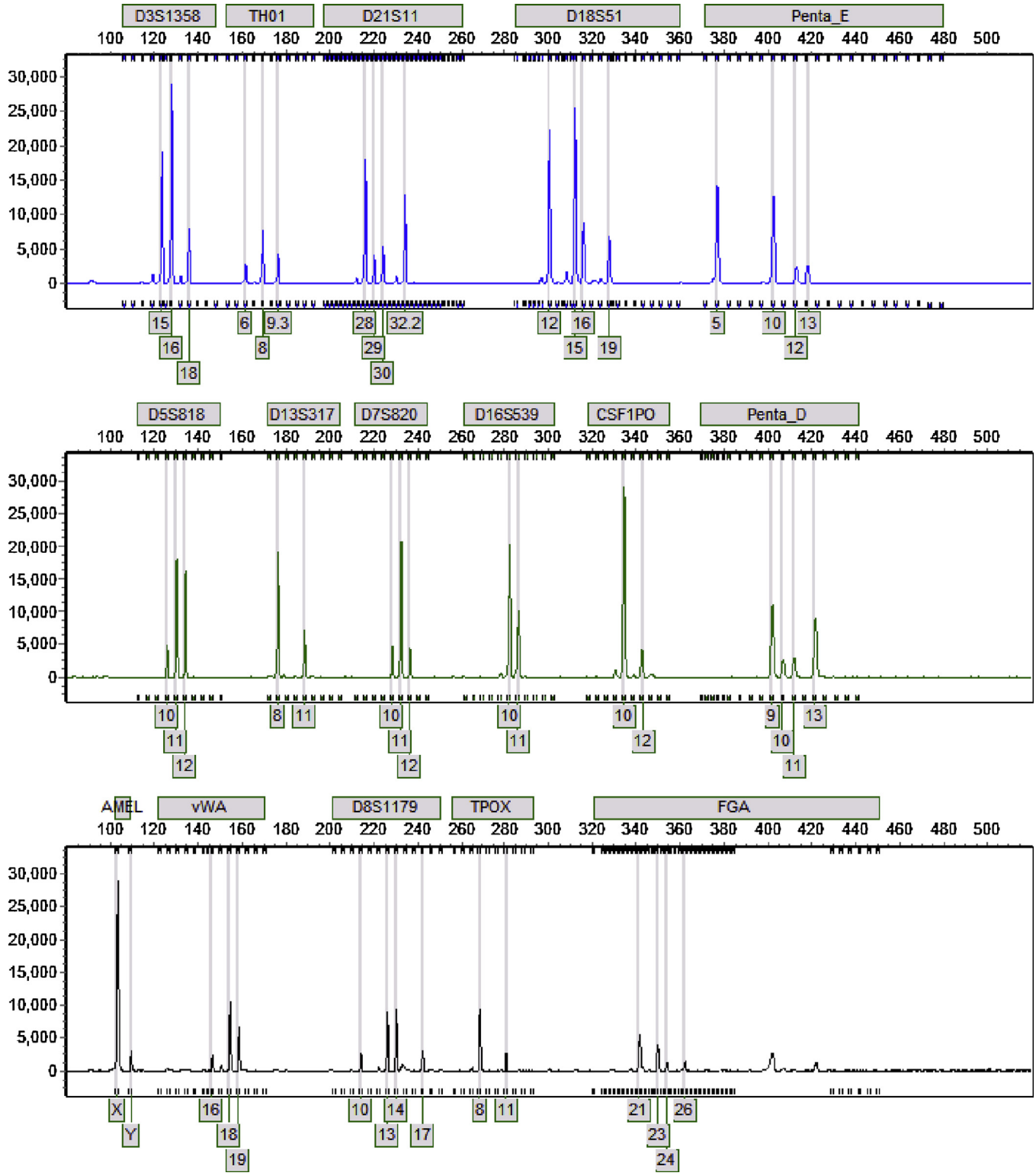

Fig. 13. RapidHIT System STR profile of NIST SRM 2391c component D.

represent the standard deviation of average peak height derived from the three repeat runs on a system using the same reagent lot. The lot-to-lot variation in average peak height is within the expected variation from repeat runs within a single lot, indicating there are no significant lot-to-lot reproducibility issues.

3.6. Species specificity

A species specificity study was performed to verify previous published results [31]. Five animal genomic DNAs and mixtures of pooled microbial organisms were tested for cross-reactivity using

both the standard bench methods as well as the RapidHIT System. Non-human DNAs (10 ng each from bovine, chicken, horse, porcine, rabbit) and a pooled mixture of the most common microbial organisms found in the oral cavity (ca. 10^5 copies each from *Fusobacterium nucleatum*,*Lactobacillus casei*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus salivarius*, *Enterococcus faecali,* ATCC) were subjected to PCR amplification (in triplicate) on the 9700 using a 20 μL total reaction volume for 30 cycles with separation on the 3130xL and data analysis was performed.

For PCR amplification on the RapidHIT System each non-human species was added to the premix reagents (8 ng/20 μL) prior to amplification. Three replicates were amplified alternating the position of each species in a channel for each run. Five replicates of the pooled mixture of microbial DNA were added to the premix reaction (~300,000 genome copies of each/20 μL) and run on one instrument (Supplemental Fig. S9). Ensenberger et al. [32] has previously reported detection of peaks in the area below the amelogenin locus in some non-human animal samples during their developmental validation of the PowerPlex16HS system. Similarly, at this high non human DNA input load, reproducible peaks were detected in bovine (TMR ~99 bp), chicken (JOE ~437–443 bp), horse (TMR ~99 bp), pork (TMR ~100 bp) and rabbit (FL ~112 bp) DNAs tested on both the 9700 thermal cycler/3130xl system (data not shown) and the RapidHIT System (Supplemental Fig. S9).

Non-human DNA or the microbial pool was also added to cotton swabs containing a quantified amount of human salvia cells from a male donor prior to insertion into the cartridge lysis chamber. The amount of species DNA applied to the swab was increased to 50 ng and 100 ng for non-human animal DNA and ~10^6 copies (per microorganism) to simulate acquisition during buccal swab collection. When the non-human DNA was applied to swabs containing human saliva cells, the presence of exogeneous DNA, e.g., chicken, rabbit, horse (Fig. 12), or bovine, pork, microbial (data not shown) did not produce peaks in the size range for human alleles. Horse DNA produced a reproducible peak that migrates below the shortest marker.

3.7. Mixtures results

To characterize the performance of the RapidHIT System with mixtures, the NIST component D mixture was analyzed (Fig. 13). After manual review and interpretation of the profiles using the interpertation guidelines (Supplemental Table S1), all alleles were successfully identified. The peak heights for alleles present in the mixture were roughly consistent with the 3:1 ratio of component A to component C.

In addition, a mixture study was performed to determine the mixture ratio at which a minor contributing profile could be detected in the presence of a major contributor profile. Mixtures were prepared using cells harvested from the saliva of a male and a female donor, quantified by qPCR, and mixed together to create a range of mixtures with the total amount of DNA on the swab constant with the male DNA representing the minor component. When analyzed on the RapidHIT System, 19/19 non-overlapping alleles were called for the minor contributing profile at a major: minor ratio of 1.5:1 and at 2.3:1. 18/19 alleles were called at a major:minor ratio of 4:1 (Supplemental Fig. S10) and 8/19 alleles were called at a major:minor ratio of 9:1.

These results demonstrate that the PowerPlex 16 RapidHIT chemistry run on the RapidHIT System can successfully separate mixtures by capillary array electrophoresis. While this developmental validation was designed for single source reference samples, the results shown in Supplemental Fig. S10, where only a single allele of a 4:1 mixture had dropped out, combined with Fig. 13 results, suggest that mixtures are successfully extracted, amplified, and separated at major:minor ratios of about >3:1.

4. Conclusions

The RapidHIT System was developmentally validated for fully automated sample-to-profile processing of reference samples with manual review by analysts of the profiles. Data presented from this developmental validation demonstrates that the system produces reproducible, high quality results with 100% concordant calls for all buccal reference samples analyzed with full profiles. The success rate of producing full profiles for buccal samples was 88%.

The data presented demonstrates the RapidHIT System is robust to moderate variations to the optimum protocol, including variation in the lysis, extraction, amplification, and separation parameters. The system produces comparable, high quality data across different reagent lots and instruments. The STR profiles are highly specific for human DNA sequences and are not sensitive to the presence of a variety of amplification inhibitors. The sensitivity of the RapidHIT System matches the requirements for analysis of buccal samples in forensic laboratories allowing for significant differences in collection protocols and buccal cell collection efficiencies.

The system performance for buccal swabs with the PowerPlex 16 HS Rapid chemistry has been tested in an interlab study conducted at the FBI Laboratory, NIST, and USACIL with similar results to those reported here, also with concordant profiles [33]. In additional studies, the RapidHIT System has been shown to achieve similar performance for reference samples using the PowerPlex 16 HS RapidHIT chemistry in an internal validation [34] with first pass success rates comparable to laboratory methods [35].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2014.12.004.

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